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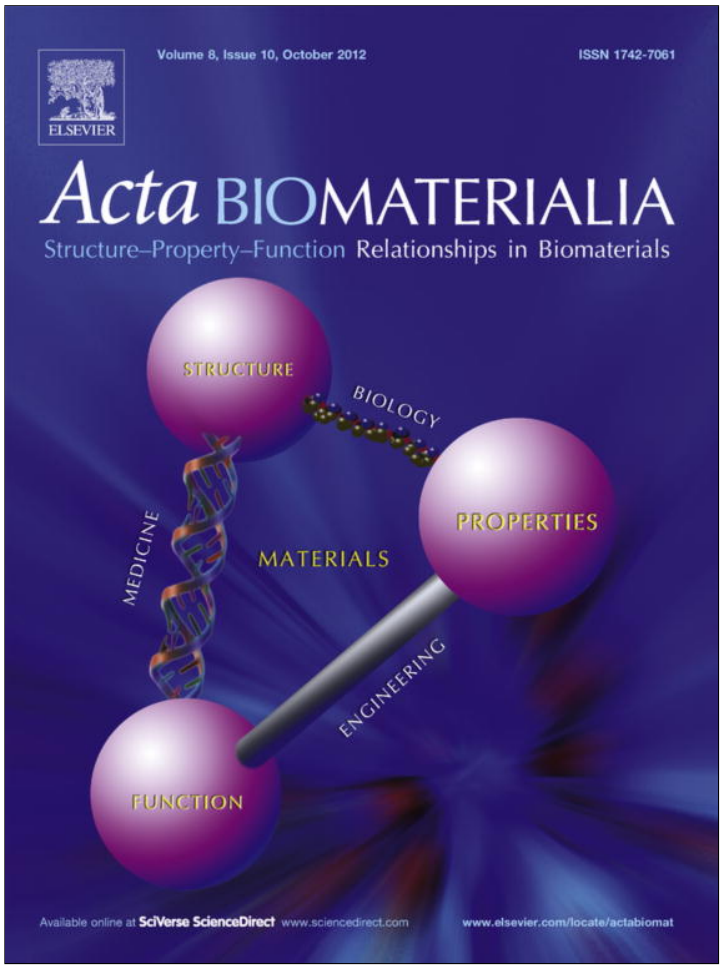

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Chemistry-dependent adsorption of serum proteins onto polyanhydride microparticles differentially influences dendritic cell uptake and activation

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ABSTRACT

The delivery of antigen-loaded microparticles to dendritic cells (DCs) may benefit from surface optimization of the microparticles themselves, thereby exploiting the material properties and introducing signals that mimic pathogens. Following in vivo administration microparticle surface characteristics are likely to be significantly modified as proteins are quickly adsorbed onto their surface. In this work we describe the chemistry-dependent serum protein adsorption patterns on polyanhydride particles and the implications for their molecular interactions with DCs. The enhanced expression of MHC II and CD40 on DCs after incubation with amphiphilic polyanhydride particles, and the increased secretion of IL-6, TNF-α, and IL-12p40 by hydrophobic polyanhydride particles exemplified the chemistry-dependent activation of DCs by sham-coated particles. The presence of proteins such as complement component 3 and IgG further enhanced the adjuvant properties of these vaccine carriers by inducing DC maturation (i.e. increased cell surface molecule expression and cytokine secretion) in a chemistry-dependent manner. Utilizing DCs derived from complement receptor 3-deficient mice ($CR3^{-/-}$ mice) identified a requirement for CR3 in the internalization of both sham- and serum-coated particles. These studies provide valuable insights into the rational design of targeted vaccine platforms aimed at inducing robust immune responses and improving vaccine efficacy.

1. Introduction

The design of vaccine adjuvants capable of activating innate immunity is critical for the induction of protective immune responses [1,2]. A key step in the activation of the innate immune system is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the surface of antigen-presenting cells (APCs), including dendritic cells (DCs) [1–3]. DCs can internalize and process soluble antigens, resulting in interactions with other immune cells, such as naïve T cells [2,4,5]. The use of polymer particles to deliver antigen, either encapsulated or bound to the surface, has been shown to enhance antigen presentation compared with the administration of soluble antigen alone [6–8].

The interaction of antigen-loaded microparticles with DCs may benefit from engineering the microparticle surface by exploiting the material properties and introducing motifs that mimic pathogens [9]. For example, it has been demonstrated that cationic surfaces greatly enhance uptake [10]. On the other hand, the presence of certain ligands which bind to specific cellular receptors promotes internalization [3,4,11]. After contact with serum the particles undergo significant changes in their surface properties because of the rapid adsorption of serum proteins [12,13].

Polyanhydride microparticles have been shown to possess immunomodulatory properties [14,15] which, when combined with their ability to stabilize and provide sustained release of protein antigens [16–20], makes them excellent vaccine adjuvants. Our previous work has demonstrated that serum protein adsorption patterns on polyanhydride microparticles are correlated with their surface characteristics (i.e. hydrophobicity), suggesting that the adsorption of serum proteins can be tailored by controlling the particle surface chemistry [13]. Immunoglobulin G (IgG), complement factors, and other proteins (i.e. opsonins) that have been identified on the surface of microparticles likely influence particle uptake by APCs [13,21,22]. Indeed, pathogens such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Mycobacterium leprae* coat themselves with serum proteins [23–26]. Opsonization of the pathogen facilitates host cell phagocytosis by promoting interactions with specific cell surface receptors, including complement, Fcγ, and mannose receptors [23–28]. Therefore, understanding the biological consequences of serum protein adsorption onto particles and its effect on APC activation may provide vital insights for the rational design of improved biomaterial-based adjuvants.

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This study was designed to investigate the differential adsorption of mouse serum proteins onto the surface of polyanhydride microparticles and to understand the effects of protein adsorption on uptake by and activation of DCs. Polyanhydrides based on sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Fig. 1) were evaluated in this work. The profile of serum proteins adsorbed onto the surface of the polyanhydride particles was indeed found to be influenced by the polymer chemistry and subsequently promoted differential effects on DC activation. Moreover, complement receptor 3 (CR3)-mediated pathways were determined to be critical for the internalization of polyanhydride particles by DCs.

2. Materials and methods

2.1. Materials

The chemicals needed for monomer synthesis and polymerization, sebacic acid (99%), p-carboxy benzoic acid (99%), and 1-methyl-2-pyrrolidinone anhydrous (99%), were purchased from Aldrich (Milwaukee, WI); 4-p-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2 pyrrolidinone, and triethylene glycol were purchased from Sigma Aldrich (St Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Stockport, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). Materials for SDS-PAGE and two-dimensional (2-D) electrophoresis, which included 12% Tris–glycine precast gels, unstained protein standards, Flamingo gel stain, 11 cm immobilized pH gradient (IPG) strips (pH 3–10, nonlinear), and 4–15% polyacrylamide gels were purchased from BioRad Laboratories (Richmond, CA). Phosphatase substrate was purchased from Aldrich (St Louis, MO). β-Mercaptoethanol, *Escherichia coli* lipopolysaccharide (LPS) O111:B4, and rat immunoglobulin (rat IgG) were purchased from Sigma Aldrich. Materials required for the DC culture medium included: granulocyte–macrophage colony-stimulating factor (GM-CSF), purchased from PeproTech (Rocky Hill, NJ); HEPES buffer, RPMI 1640, penicillin/streptomycin, and L-glutamine, purchased from Mediatech (Herndon, VA); heat inactivated fetal bovine serum, purchased from Atlanta Biologicals (Atlanta, GA). Materials used for flow cytometry included: BD stabilizing fixative solution purchased from BD Bioscience (San Jose, CA); unlabeled anti-CD16/32 FcγR, purchased from Southern Biotech (Birmingham, AL); unlabeled hamster IgG, fluorescein isothiocyanate (FITC)-conjugated anti-mouse MHC II (I-A/I-E) (clone M5/114.15.2), PE-conjugated anti-mouse MHC Class I (H-2Kd/H-2Dd) (clone 34-1-2S), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), phycoerythrin-Cy7 (PE/Cy7) anti-mouse CD86 (clone GL-1), Alexa Fluor® 700 anti-mouse CD11c (clone N418) and the corresponding isotypes, FITC-conjugated rat IgG2bκ, PE-conjugated rat IgG2a (clone eBR2a), APC rat IgG2aκ (clone eBR2a), PE/Cy7-conjugated rat IgG2b (clone KLH/G2b-1-2), Alexa Fluor® 700-conjugated Armenian hamster IgG (clone eBio299Arm), all purchased from eBioscience. Cadmium selenide quantum dots (QDs) (emission at 630 nm) were a kind gift from Dr. Aaron Clapp of Iowa State University.

2.2. Monomer and polymer synthesis

Diacids of CPH and CPTEG were synthesized as described previously [29,30]. SA and CPH prepolymers were synthesized by the methods described by Shen et al. [31] and Conix [29], respectively. Subsequently 50:50 CPH:SA and 50:50 CPTEG:CPH co-polymers were synthesized by melt polycondensation as described by Kipper et al. [32] and Torres et al. [30], respectively. ^{1}H NMR spectroscopy was used to characterize the polymer structure, and the resultant spectra were consistent with previous work [30,32]. The synthesized 50:50 CPH:SA co-polymer had a M_w of 12 kDa with a polydispersity index (PDI) of 2.0, while the 50:50 CPTEG:CPH co-polymer had a M_w of 8 kDa with a PDI of 1.8. These values were obtained from ^{1}H NMR and corroborated with GPC and are consistent with previous works [8,30,32].

2.3. Microparticle fabrication and characterization

Cryogenic atomization was used to fabricate 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles, as described elsewhere [13,16,19,20]. Briefly, 100 mg of the polymer was weighed and dissolved in methylene chloride. For QD-loaded microparticles, QDs were added to the dissolved polymer and dispersed by sonication at 40 Hz for 30 s. The polymer solution was then pumped through an 8700–1200 MS ultrasonic atomizing nozzle (SonoTek Corp., Milton, NY) into 200 ml of frozen ethanol (with an excess of liquid nitrogen). Microparticles were fabricated at 4 °C. Compositions were stored at −80 °C for 3 days. For 50:50 CPTEG:CPH, after the first 24 h 200 ml of cold ethanol was added to reduce aggregation, the solutions were stirred at 300 r.p.m. for 15 min and placed back in the freezer at −80 °C. After 3 days vacuum filtration was used to collect the microparticles and they were dried overnight under vacuum. Scanning electron microscopy (SEM) (JEOL 840 A, JEOL Peabody, MA) was used to observe the morphology of the micro-

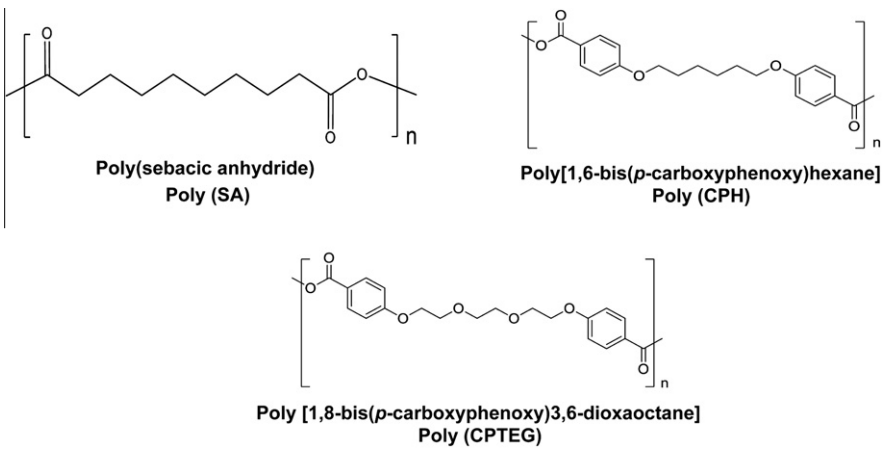

Fig. 1. Chemical structures of the SA, CPH, and CPTEG repeat units. Upon degradation these polyanhydrides produce dicarboxylic acids in which the anhydride bonds are replaced by –COOH groups on both ends.

particles. The particle size distribution was obtained from SEM images using ImageJ image analysis software (National Institutes of Health, Bethesda, MD) [13,16,19,20]. An average of 200 particles per image were analyzed. Quasi-elastic light scattering (QELS) was used to determine the ζ-potential of the particles, as described previously [3]. Particle morphology and size were consistent with previous works [14,16–20]. An average size of 6 ± 4 μm and 5 ± 3 μm was obtained for the 50:50 CPH:SA and 50:50 CPTEG:CPH particles, respectively.

2.4. Mice

C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) and $CR3^{-/-}$ mice (C57BL/6 background) were a generous gift from Dr. Mary Ann McDowell of the University of Notre Dame. All mice were housed under specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

2.5. Adsorption of mouse serum onto microparticles

To facilitate the adsorption of serum proteins, 13.3% w/v suspensions of polymer microparticles were prepared in 0.1 M phosphate-buffered saline, pH 7.4 (PBS). Mouse serum was obtained via cardiac puncture of killed C57BL/6 mice and serum was allowed to clot overnight at 4 °C. Clarified serum was collected by centrifugation and stored in aliquots at −20 °C until use. Particle suspensions were incubated with mouse serum in a 4:1 final volume ratio of particle suspension to serum (i.e. 25% serum) to obtain serum-coated particles. Control particles were incubated in PBS without mouse serum (i.e. sham-coated particles). The suspension was mildly vortexed for 1 min and incubated for 30 min at 37 °C. After incubation the particle suspension was centrifuged at 12,000*g* for 10 min to pellet the particle–protein complexes. The pellet was resuspended in PBS, transferred to a new vial, and centrifuged again (under the same conditions) to pellet the particle–protein complexes. This procedure was repeated three times. After the third washing step the supernatant did not contain any detectable protein based on a micro BCA assay. Microparticles were dried under vacuum for at least 2 h.

2.6. Determination of protein adsorption patterns

2-D electrophoresis was used to analyze the proteins adsorbed onto polyanhydride particles. Similar amounts of serum-coated particles of the different chemistries were incubated with 250 μl of elution buffer (10 wt.% SDS and 2.3 wt.% dithioerythritol) to elute bound proteins from the polyanhydride particles. Samples of serum-coated particles were heated at 95 °C for 10 min, the particles were separated from the eluted proteins by centrifugation for 10 min at 12,000*g*, and the supernatants used to perform 2-D electrophoretic analysis [13,16]. The use of this elution protocol removed most of the adsorbed protein, as verified by micro BCA and SDS–PAGE analysis of the particles after the elution step. It is important to note that the particles were only heated to elute the adsorbed proteins from the particles prior to performing the electrophoretic analysis. The elution step, including heating of the serum-coated particles, was only performed to characterize the patterns of adsorbed proteins and not for the particles used in cell studies.

To analyze the eluted proteins by 2-D electrophoresis equal volumes of serum protein-containing elution buffers were loaded onto the gels and the first dimensional separation was performed in an IPGPhor system (GE Healthcare, Piscataway, NJ) using 11 cm IPG strips (pH 3–10) following a slow voltage ramping protocol: 50 V for 10 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h [13,33]. For the second dimension of the separation analysis the IPG strips were loaded on 4–15% polyacrylamide gels and run for 2 h at 140 V. The gels were incubated in fixative solution (40% ethanol, 10% acetic acid) at 4 °C for 3 h. Next they were stained with fluorescent flamingo gel stain (BioRad Laboratories, Richmond, CA) overnight, and washed with a 0.01% Tween 20 solution to reduce non-specific fluorescence [13,16]. A Typhoon 8600 (GE Healthcare, Piscataway, NJ) fluorescence scanner was used to obtain images of the gels. The experiments were performed in triplicate. A qualitative analysis was performed by comparing the gels of the eluted proteins with those obtained from mouse sera. Progenesis SameSpots software (Nonlinear Dynamics Inc., Durham, NC) was used to identify the main bands on the gels by comparison with their molecular weight and isoelectric point position on the gel. The fluorescence intensity of each protein spot was obtained using ImageQuantTL (GE Healthcare, Piscataway, NJ) and normalized to the total fluorescence intensity of the proteins in the gel. Data for each protein spot is presented as a percentage of the total fluorescence intensity.

2.7. Identification of complement components C3 and C3a

Sandwich ELISA was used to identify specific components in the protein mix recovered from the microparticle surfaces. A mouse complement component C3 ELISA kit from Kamiya Biomedical Company (Seattle, WA) was utilized as per the manufacturer's recommendations for quantitative determination of C3 in protein samples eluted from particle surfaces. Complement activation was assessed by measuring the concentration of C3a in serum supernatants after incubation with polyanhydride particles (C3a ELISA kit, Kamiya).

2.8. DC culture and stimulation

DCs were grown as described previously [3,14,34] and stimulated with either 200 ng ml^{-1} LPS (positive control), 125 μg ml^{-1} QD-loaded or blank 50:50 CPH:SA or 50:50 CPTEG:CPH microparticles, or left untreated (NS) (non-simulated negative control). Treatments were applied to the DCs incubated in culture medium (RPMI containing 1% L-glutamine, 1% penicillin/streptomycin solution, 2% HEPES, 0.5% gentamicin, 0.1% β-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS) supplemented with GM-CSF (10 ng ml^{-1}) on day 9 post-harvest and incubated for 48 h. DCs were >90% positive for the DC marker CD11c. For internalization studies a QD control (background) was used to account for "false positives" due to QDs released upon particle degradation [35]. QD-loaded particles were incubated in DC culture medium for 48 h. After centrifugation the supernatants containing the released QDs were added to DCs. After 48 h the fluorescence registered for these control groups was considered the background [35]. FluoSpheres® carboxylate-modified polystyrene microspheres (PS) (2 μm, 580/605 nm, Invitrogen, Carlsbad, CA) were used as controls for the internalization experiments. It is instructive to note that the sham-coated particles represent a similar control to using complement-deficient sera because these particles were not preincubated with "fresh" mouse serum, but were in contact with heat-inactivated FBS, which forms part of the medium utilized for the culture of DCs and during the uptake and cell surface marker expression assays.

2.9. Flow cytometry analysis

Multicolor flow cytometric analysis of surface molecule expression was performed to assess expression of MHC I, MHC II, CD40,

and CD86 as previously described [3,14,34,35]. Samples were acquired in a Becton-Dickinson FACSCanto™ flow cytometer (San Jose, CA) and the data analyzed using FlowJo (TreeStar Inc., Ashland, OR).

2.10. Cytokine assays

After stimulation for 48 h with nanoparticles the cell-free supernatants were assayed for IL-1β, IL-10, TNF-α, IL-6, and IL-12p40 using a multiplex cytokine assay in conjunction with a Bio-Plex 200 System (BioRad, Hercules, CA).

2.11. Statistical analysis

The statistical software JMP®7 was used to analyze the cell surface marker, cytokine, and internalization data. One-way ANOVA and Tukey's HSD were used to determine statistical significance among treatments and $P < 0.05$ was considered significant.

3. Results

3.1. Chemistry-dependent adsorption of immunoglobulin G (IgG) and complement component C3 on polyanhydride particles

It is known that the surface charge of polymeric particles can influence their uptake by phagocytic cells [36]. Measurements of particle ζ-potential using quasi-elastic light scattering have resulted in similar values (-22 ± 5.5 mV) for all particles regardless of chemistry and these values are consistent with previous work [3]. The presence of deprotonated carboxylic groups may account for the negative surface charge of the polyanhydride particles. After incubation with mouse serum the average ζ-potential increased to -5 ± 6 mV, indicative of adsorption of serum proteins onto the particle surface. This value is consistent with other work in which BSA adsorbed onto negatively charged polystyrene particles reduced their highly negative ζ-potential [22].

A BCA assay was used to quantify the total amount of eluted proteins from the microparticle surfaces. This analysis revealed that protein adsorption onto 50:50 CPH:SA particles (11.5 ± 1.3 μg mg particles^{-1}) was greater than that adsorbed onto 50:50 CPTEG:CPH particles (4.6 ± 0.5 μg mg particles^{-1}). These findings are consistent with previous work demonstrating that hydrophobic CPH:SA particles readily adsorb more proteins onto their surface [13]. In contrast, the presence of ethylene glycol (EG) motifs in the CPTEG:CPH co-polymer particles leads to reduced protein adsorption [13,37]. This differential protein binding occurs even when both chemistries showed similar initial ζ-potential values, indicating that surface charge alone does not dictate protein adsorption. As indicated in Fig. 1, the EG motifs in CPTEG are distributed throughout the bulk of the particles (i.e. not simply on the surface). This distribution influences the hydrophobicity of the CPTEG:CPH particles without having a direct charge shielding influence on the electrokinetic potential of the particles, as indicated by the ζ-potential measurements.

The effect of poly(ethylene glycol)-containing formulations on the reduction in serum protein adsorption has been demonstrated previously, with a particular focus on the reduced adsorption of IgG and C3 [37]. Fig. 2A and B shows representative images of 2-D gels depicting the profiles of proteins eluted from 50:50 CPH:SA and 50:50 CPTEG:CPH particles, respectively. In order to identify the specific proteins adsorbed onto the microparticles the 2-D gels were compared with a reference map and databases of mouse serum [38]. Albumin and IgG were identified as the most abundant proteins adsorbed onto both 50:50 CPH:SA (Fig. 2A) and 50:50 CPTEG:CPH (Fig. 2B) particles. Differential patterns of protein adsorption were also observed to be chemistry dependent. For example, transferrin and several apolipoproteins bound to and were eluted from 50:50 CPTEG:CPH (Fig. 2B) particles but not from 50:50 CPH:SA particles (Fig. 2A). Encapsulation of Ova did not change the chemistry-dependent pattern of proteins adsorbed onto the microparticles (data not shown). A detailed analysis of all the principal proteins eluted from the surface of polyanhydride microparticles is presented in Table 1 in terms of the percentage of total amount of proteins adsorbed onto the particles (based on the fluorescence intensity, as described in Section 2). This semi-quantitative approach is in contrast to an immunoblot analysis in which antibodies are used for the detection of specific proteins. Our use of a serum protein database enabled a comparison between the profile of proteins adsorbed onto the surface of different particle chemistries and has been previously used to study serum protein adsorption on a variety of biomaterial surfaces [39,40].

Of particular note, complement component C3 was observed predominantly on the surface of 50:50 CPH:SA particles (Fig. 2A) but not on 50:50 CPTEG:CPH particles (Fig. 2B). A protein with a molecular mass and isoelectric point of 75 kDa and 6.4, respectively, was identified as the β-chain of C3. A second complement component with a lower molecular weight matched with a more acidic fragment of C3 (C3e) in the protein database. An ELISA analysis confirmed that significantly more C3 was adsorbed onto the surface of 50:50 CPH:SA particles compared with 50:50 CPTEG:CPH particles (Fig. 2C, 0.12 and 0.03 μg cm^{-2}, respectively). To characterize the chemistry-dependent patterns of complement activation C3a was evaluated as evidence of C3 cleavage. Greater amounts of C3a were detected in the serum supernatants after incubation with 50:50 CPH:SA particles (Fig. 2D). Together these data demonstrate that 50:50 CPH:SA particles bind and activate more C3 than do 50:50 CPTEG:CPH particles.

3.2. Internalization of sham- and serum-coated polyanhydride particles mediated by complement receptor 3

The data presented in Fig. 2 suggest that 50:50 CPH:SA particles may have a greater capacity for opsonization, and subsequent cellular internalization, than 50:50 CPTEG:CPH particles. To that end we tested the ability of polyanhydride particles in the presence or absence of serum to be internalized by DCs. When sham-coated 50:50 CPH:SA particles were incubated with DCs ~20% of cells were observed to contain particles, while incubation of sham-coated 50:50 CPTEG:CPH particles with DCs resulted in less than 10% particle-positive cells (Fig. 3). The observation that DCs internalize CPTEG:CPH particles less readily is consistent with previous studies in our laboratories [9,35]. When serum-coated microparticles were added to the DC cultures 20% more DCs internalized 50:50 CPH:SA particles, presumably because of the interaction of specific serum proteins with cellular receptors, including complement receptors [6,41]. To evaluate this hypothesis we tested the internalization of sham- and serum-coated particles by DCs deficient in complement receptor 3 (CR3), a pattern recognition receptor that binds various molecules on pathogen surfaces, including complement-derived opsonins (i.e. iC3b). Engaging this receptor promotes phagocytosis of the pathogen by DCs or macrophages. Strikingly, internalization of both sham- and serum-coated polyanhydride particles was significantly reduced, regardless of particle chemistry. Similar levels of internalization of polystyrene (PS) particles (control) by wild type (WT) and CR3$^{-/-}$ DCs were observed (Fig. 3). These data demonstrate that CR3 is required for DC internalization of polyanhydride microparticles, but not PS microparticles of similar size.

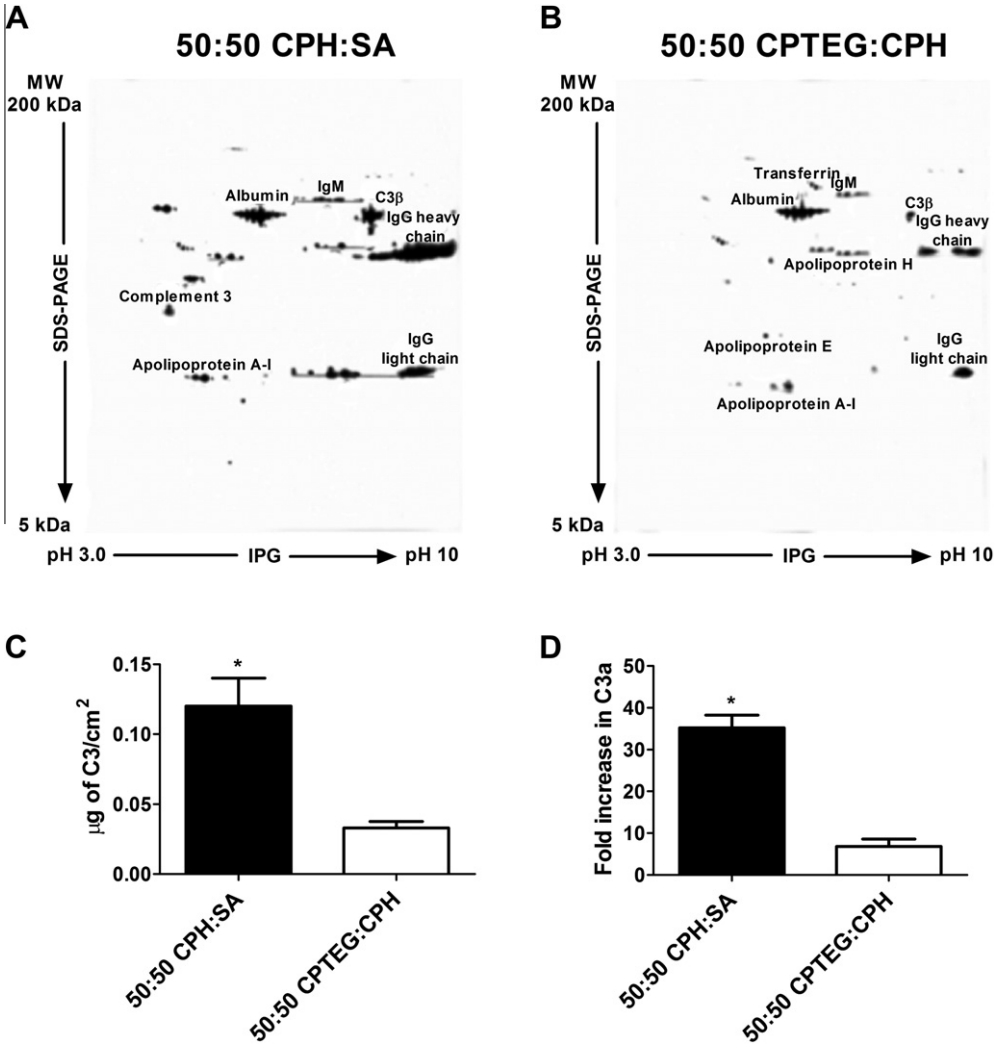

Fig. 2. Chemistry-dependent adsorption patterns of immunoglobulin G (IgG) and complement component C3 on polyanhydride particles. Representative 2-D gels of proteins adsorbed onto (A) 50:50 CPH:SA and (B) 50:50 CPTEG:CPH particles. (C) Complement component C3 was adsorbed onto the surface of 50:50 CPH:SA microparticles, as measured via an anti-mouse C3 ELISA. Data are presented as the means ± SEM of three independent experiments. (D) 50:50 CPH:SA microparticles induced C3 cleavage (i.e. complement activation), as measured by the appearance of C3a in mouse serum supernatants after incubation with the microparticles. Data are normalized to control serum that was similarly incubated in the absence of any particles (negative control). Data are presented as the means ± SEM of three independent experiments. (C and D) *Statistically significant difference between the two chemistries at $P < 0.05$.

3.3. Serum proteins adsorbed onto particle surfaces influence the expression of MHC II and co-stimulatory molecules on DCs in a chemistry-dependent manner

We have previously demonstrated chemistry-dependent activation of DCs by polyanhydride particles [14,35]. The results shown in Fig. 4A corroborate these findings. Specifically, enhanced expression of MHC II over non-stimulated DCs was observed when DCs were co-cultured with sham-coated 50:50 CPTEG:CPH particles, but not with sham-coated 50:50 CPH:SA particles. Both chemistries equally enhanced the surface expression of the co-stimulatory molecules CD86 and CD40 (Fig. 4B and C). Compared with sham-coated microparticles, expression of both MHC II and CD40 was up-regulated when DCs were incubated with serum-coated 50:50 CPH:SA particles (Fig. 4A and C).

A comparative analysis of WT DCs that had internalized microparticles versus those that had not internalized particles showed a direct correlation between particle internalization and the surface expression of CD40. In contrast, enhancement of DC MHC II and CD86 surface expression was not dependent on particle internalization (Supplementary Fig. S1).

3.4. CR3 is required for DC internalization of both sham- and serum-coated particles as well as up-regulation of MHC II and CD40 expression

The data shown in Fig. 5 demonstrate that $CR3^{-/-}$ DCs present a different DC activation profile compared with WT DCs. In contrast to WT DCs, $CR3^{-/-}$ DCs did not up-regulate the expression of either MHC II or CD40 after stimulation with either sham- or serum-coated particles (Fig. 5A and C). The expression levels for these two markers on $CR3^{-/-}$ DCs were not statistically significantly different from the levels obtained for non-stimulated cells. In addition, the expression of CD86 on $CR3^{-/-}$ DCs was not statistically significant different from that observed on WT DCs for all the particle groups, indicating that its expression is independent of CR3-mediated internalization (Fig. 5B).

3.5. Serum proteins adsorbed onto 50:50 CPTEG:CPH microparticles enhance secretion of pro-inflammatory cytokines

After 48 h incubation with microparticles of either chemistry enhanced DC secretion of IL-1β was observed (Fig. 6A). However,

Table 1
Percentages of the most abundant protein species adsorbed onto 50:50 CPH:SA and 50:50 CPTEG:CPH particles[a,b].

Adsorbed protein	50:50 CPH:SA	50:50 CPTEG:CPH
Albumin	24.8 ± 0.5	27.2 ± 0.3
IgG γ chain	35.4 ± 1.1	25.0 ± 0.6
IgG light chain	4.5 ± 0.4	4.8 ± 1.0
IgM	7.3 ± 0.2	9.8 ± 0.5
Complement component C3	23.4 ± 1.9	8.4 ± 0.5
Apolipoprotein A-I	1.4 ± 0.1	2.6 ± 0.3
Apolipoprotein E	0.3 ± 0.1	3.5 ± 0.4
Apolipoprotein H	0.0 ± 0.0	7.5 ± 1.1
Transferrin	0.0 ± 0.0	2.3 ± 0.2

[a] Data for each protein spot are presented as percentage fluorescence intensity. The fluorescence intensity of each protein spot was quantified using ImageQuantTL and normalized to the total fluorescence intensity of the gel, which included all protein spots.
[b] Data is the average of three independent experiments.

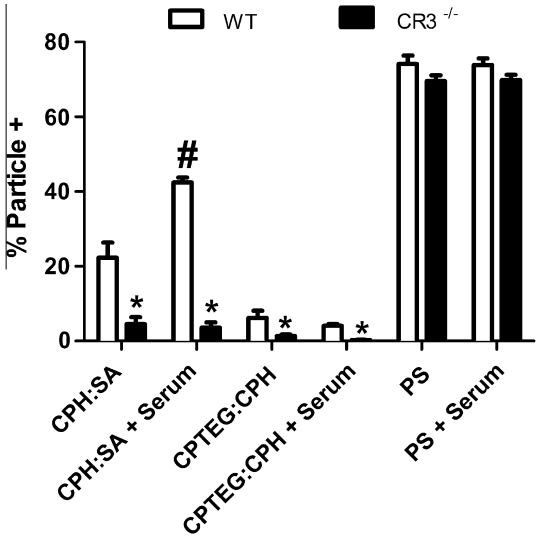

Fig. 3. Internalization of sham- and serum-coated polyanhydride particles was mediated by complement receptor 3. Percent WT (□) and CR3$^{-/-}$ (■) DCs that internalized sham- or serum-coated microparticles after 48 h. Data are expressed as the means ± SEM of three independent experiments performed in triplicate. *Statistically significant difference between WT and CR3$^{-/-}$ DC within a treatment at $P < 0.05$. #Statistically significant difference between sham- and serum-coated particles within a chemistry at $P < 0.05$.

only sham-coated 50:50 CPH:SA particles increased the secretion of IL-6, IL-12p40, and TNF-α over non-stimulated cells compared with sham-coated 50:50 CPTEG:CPH particles (Fig. 6B–D), which is consistent with previous observations [14,34]. Interestingly, the secretion of IL-6, IL-12p40, and TNF-α by DCs co-cultured with serum-coated 50:50 CPTEG:CPH particles was significantly elevated compared with sham-coated 50:50 CPTEG:CPH particles (Fig. 6B–D).

In general no differences were observed in the amount of cytokines secreted by WT or CR3$^{-/-}$ DCs. The only exception was a lack of increase in the production of IL-12p40 by CR3$^{-/-}$ DCs when cultured with serum-coated 50:50 CPH:SA particles, as was observed for WT DCs (Fig. 7). Previous work has shown that particle internalization is related to enhanced secretion of IL-12p40 [35]; this may be attributed to the observation that fewer CR3$^{-/-}$ DCs internalized 50:50 CPH:SA particles than WT DCs. However, the enhanced secretion of IL-12p40 may not be directly proportional to internalization, since similar concentrations of IL-12p40 are secreted by WT DCs that were stimulated with sham- and serum-coated 50:50 CPH:SA particles, even when greater internalization was observed for the serum-coated particles.

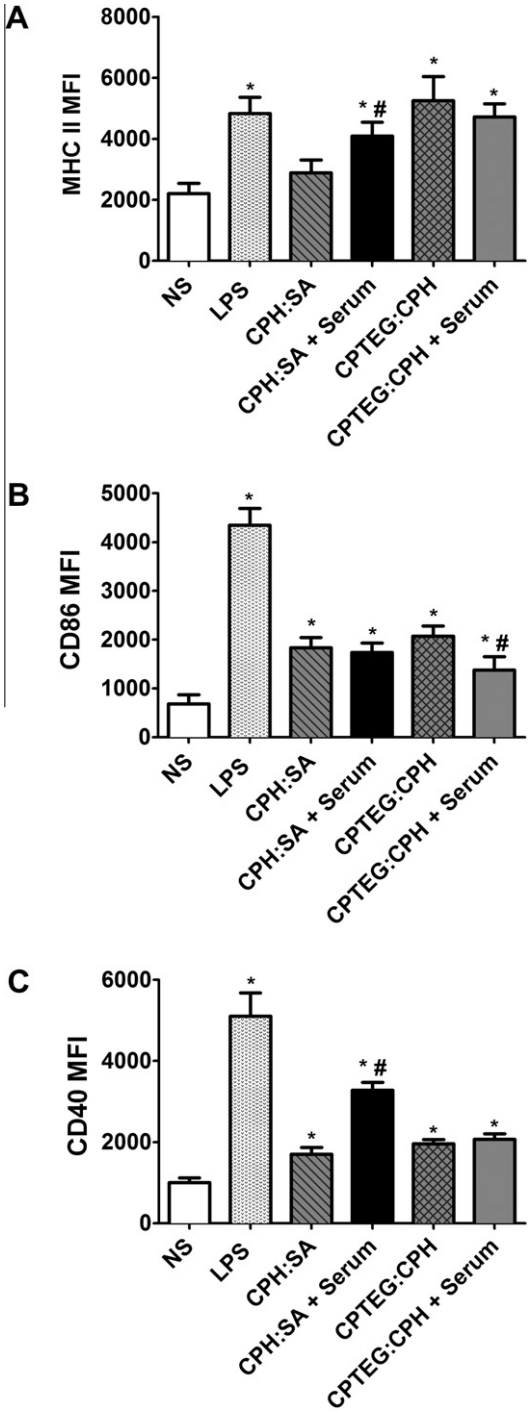

Fig. 4. Serum proteins adsorbed onto particles influenced the expression of MHC II and co-stimulatory molecules on DCs in a chemistry-dependent manner. After co-culture with either sham- or serum-coated microparticles for 48 h wild-type DCs were harvested and analyzed by flow cytometry for surface expression of (A) MHC II, (B) CD86, or (C) CD40. LPS-stimulated and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the means ± SEM of three independent experiments performed in triplicate. *Statistically significant difference from NS cells at $P < 0.05$. #Statistically significant difference between sham- and serum-coated particles within a chemistry at $P < 0.05$.

4. Discussion

Biodegradable polymeric particles have been extensively studied as carriers for the delivery of antigens and drugs [1,14,15,42,43]. The interaction of the surface of these particles with membrane-bound receptors on APCs will initiate particle up-

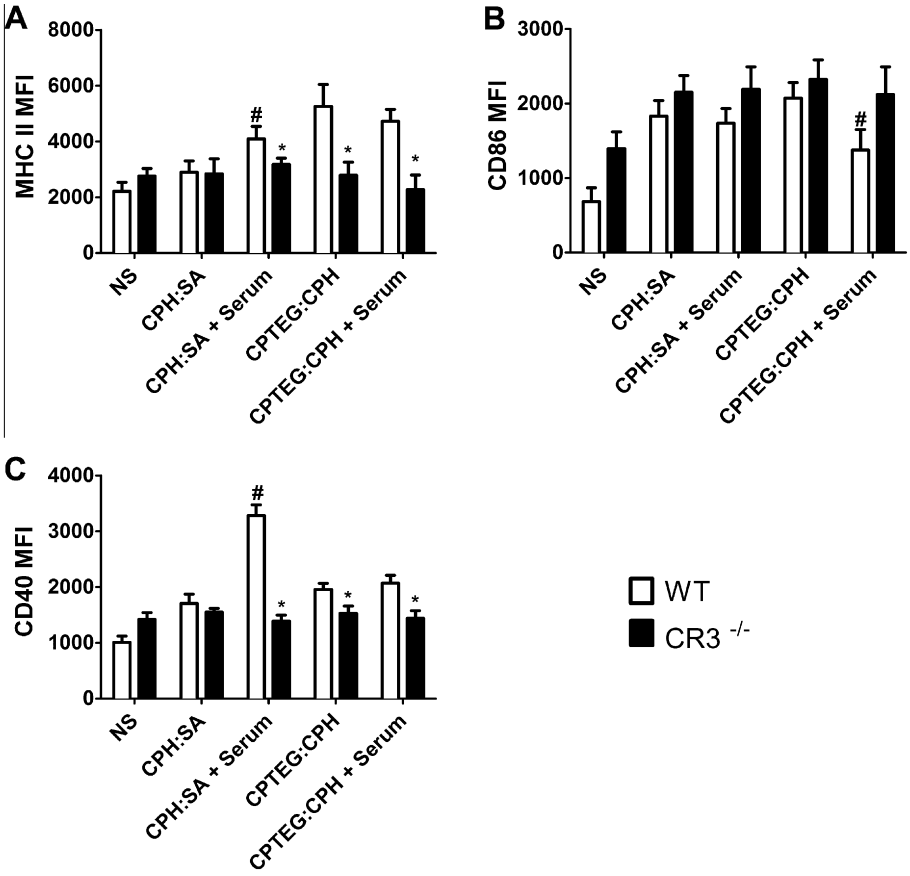

Fig. 5. CR3 is required for DC internalization of both sham- and serum-coated particles as well as up-regulation of MHC II and CD40 expression. After stimulation with either sham or serum-coated microparticles for 48 h WT (□) and CR3$^{-/-}$ (■) DCs were harvested and analyzed by flow cytometry for surface expression of (A) MHC II, (B) CD86, or (C) CD40. The expression levels for these two markers on CR3$^{-/-}$ DCs were not statistically significant from the levels obtained from non-stimulated cells. LPS-stimulated and non-stimulated cells (NS) were used as positive and negative controls, respectively. LPS was used as a positive control stimulant with mean MFI values of 4828 for MHC II, 4346 for CD86, and 5601 for CD40 for WT DCs and 4739 for MHC II, 3767 for CD86, and 3837 for CD40 for CR3$^{-/-}$ DCs. Data are expressed as the means ± SEM of three independent experiments performed in triplicate. *Significant difference between WT and CR3$^{-/-}$ DCs within a group at $P \leqslant 0.05$. #Statistically significant difference between sham- and serum-coated particles within a chemistry at $P < 0.05$.

take and influence the magnitude of the resultant immune response [1,2]. Adsorption of specific serum proteins onto the surface of polymeric particles alters their recognition and uptake by APCs [12,13,39]. The data presented herein demonstrate that chemistry-dependent adsorption of serum proteins on polyanhydride particles (Fig. 2) affects their internalization and the subsequent activation of murine DCs (Figs. 3–7 and Supplementary Fig. S1).

It is well known that protein adsorption phenomena are time dependent [13]. As reported before [13], short incubation times (<30 min) are sufficient to produce an "irreversible" layer of adsorbed proteins and for the displacement of highly abundant proteins by those with higher affinity. In this work we have sought to understand the role of protein adsorption on the uptake of particles by APCs during the early stages of the immune response. It has been previously demonstrated that these particles are rapidly internalized by APCs within 30 min [8,14,34,35], making the timescale of the current experiments consistent with the initial in vivo interactions between APCs and particles when used as vaccine adjuvants and/or antigen carriers.

Particle chemistry and hydrophobicity play integral roles in particle internalization by DCs, as well as DC activation profiles (i.e. cell surface marker expression and cytokine production) [3,8,14,34,35]. These characteristics also determine the patterns of serum protein adsorption onto particles. Specifically, hydrophobic 50:50 CPH:SA particles adsorbed more serum proteins, including IgG and complement component C3, compared with the EG-containing amphiphilic 50:50 CPTEG:CPH particles (Fig. 2). The inhibitory effect of EG-containing particles on protein adsorption has been observed previously [22,37,44,45], and it may be expected that CPTEG-containing particles will remain extracellular longer than CPH:SA particles.

The identification of C3 adsorption onto polyanhydride particles is important because fragments of this molecule are known to mediate phagocytic uptake of particles via complement receptor-mediated mechanisms [37,44]. In particular, the observation of C3 fragments on the surface of polyanhydride particles is of especial interest because it demonstrates the potential of using the complement cascade as a danger signal to stimulate innate immunity, which is a desirable goal in the design of vaccine adjuvants [46]. Complement can also promote antigen-specific immune responses by enhancing both antibody-mediated [47] and T cell-mediated immunity [48]. Just as on the surface of a pathogen, deposition of C3 on the surface of polymer particles promotes the formation of biologically active C3a and C3b fragments [46,49]. This is supported by the observation that the C3a fragment was detected in serum incubated in the presence of polyanhydride particles (Fig. 2D). Previous work by Hubbell and co-workers identified complement activation and subsequent DC activation and migration by nanoparticles containing hydroxyl end groups [50]. Hydroxyl groups are exposed when polyanhydride particles degrade and may, therefore, be involved in complement activation by promoting C3 cleavage, which is qualitatively consistent with the conclusions reported by Reddy et al. [46].

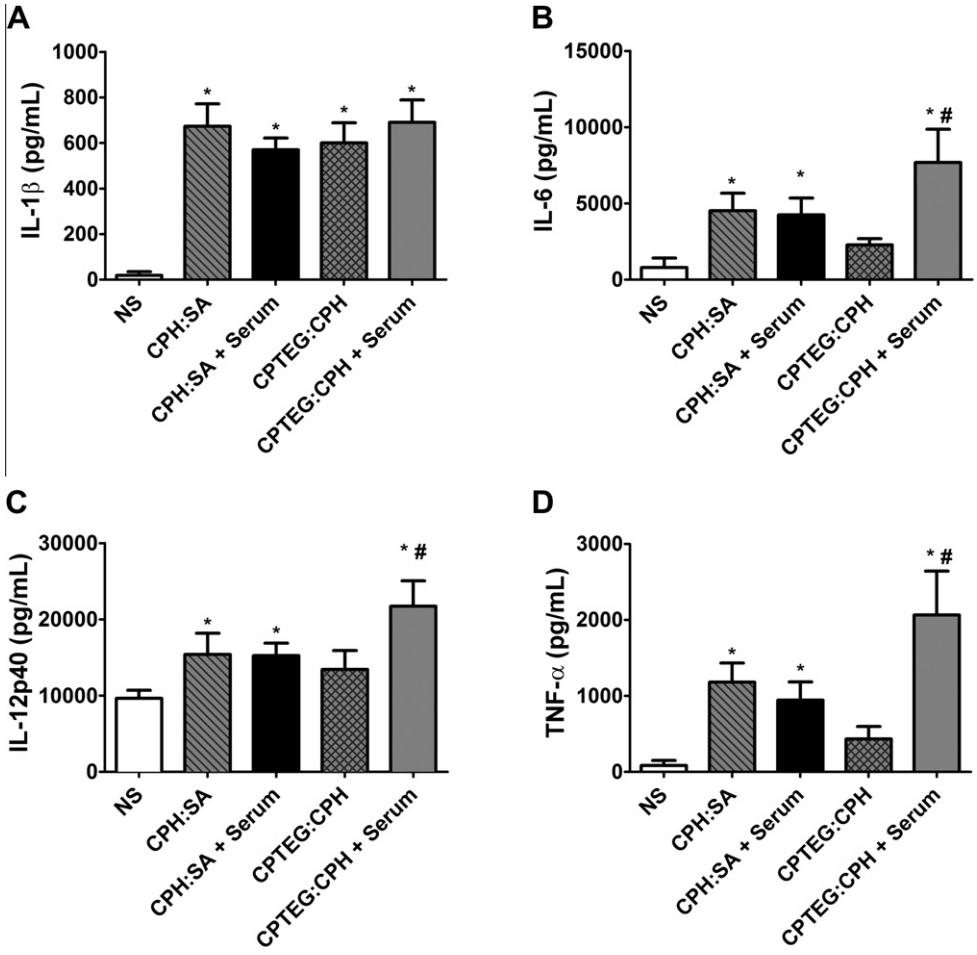

Fig. 6. Serum proteins adsorbed onto 50:50 CPTEG:CPH microparticles enhanced secretion of pro-inflammatory cytokines. After co-culture with either sham- or serum-coated microparticles for 48 h DC culture supernatants were harvested and assayed for (A) IL-1β, (B) IL-6, (C) IL-12p40, or (D) TNF-α. Data are expressed as the means ± SEM of three independent experiments performed in triplicate. LPS was used as a positive control stimulant with mean cytokine secretion values of 1041 pg ml^{-1} for IL-1β, 44,329 pg ml^{-1} for IL-6, and >50,000 pg ml^{-1} for both IL-12p40 and TNF-α. *Statistically significant difference from NS cells at $P < 0.05$. #Statistically significant difference between sham- and serum-coated particles within a chemistry at $P < 0.05$.

The molecular mechanisms governing the interactions of these particulate adjuvants with DCs has been an area of study in our laboratories [3,9,14,34,35], and we have identified polymer-associated molecular patterns and descriptors that may be responsible for the activation of DCs [9,35]. The current work builds upon these previous studies by investigating the role of serum protein adsorption and its consequent outcomes (i.e. complement activation) on the molecular interactions of particles with DCs. Sham-coated 50:50 CPH:SA and 50:50 CPTEG:CPH particles were both internalized by DCs, but a higher percentage of DCs internalized 50:50 CPH:SA particles (~22%), as previously described (Fig. 3) [8,14,35]. More DCs internalized serum-coated 50:50 CPH:SA particles (~42% of the cells were particle$^+$ cells) than sham-coated particles. This result is hypothesized to be a consequence of enhanced adsorption of the opsonins IgG and C3, which would promote interactions with the Fcγ and complement receptors, respectively [4,41]. It is known that CR3 binds to iC3b, an inactive form of the C3b cleavage fragment of C3, fixed on pathogen and particle surfaces. In addition, co-ligation of both complement and Fcγ receptors can produce cooperative effects that facilitate antibody-dependent phagocytosis [51,52]. Our data indicate that C3 deposition on the surface of 50:50 CPH:SA particles promotes the cleavage of C3 into C3a and C3b fragments (Fig. 2D), suggesting that the CR3 receptor will likely contribute to the internalization of serum-coated particles. Compared with WT DCs there were 90% fewer CR3$^{-/-}$ DCs that internalized serum-coated particles (Fig. 3). This data is consistent with previous work demonstrating the role of the CR3 receptor in the recognition of opsonized bacteria and zymosan particles [27,53,54].

In addition, CR3$^{-/-}$ DCs had a diminished capacity for uptake of sham-coated particles regardless of polyanhydride chemistry, demonstrating a CR3 receptor-mediated uptake pathway for polyanhydride particles in the absence of serum proteins (Fig. 3). These observations are consistent with the pathogen-mimicking characteristics of polyanhydride particles [9,35]. Specifically, certain domains (i.e. lectin sites) of the CR3 receptor facilitate the binding and phagocytosis of non-opsonized *M. tuberculosis*, LPS, *Leishmania* lipophosphoglycan (LPG), and various particulate saccharides, including β-glucan and zymosan [27,51,53,55]. Detailed studies in our laboratories have previously demonstrated similar DC activation phenotypes between polyanhydride particles and LPS, which were attributed to pathogen-mimicking molecular descriptors, including hydroxyl end groups and backbone oxygen moieties [9,35]. These structural descriptors may also affect the CR3-mediated internalization of polyanhydride particles described in this study, which appears to be specific for these chemistries, as CR3$^{-/-}$ DCs were able to efficiently internalize PS particles independent of serum exposure (Fig. 3).

Up-regulation of the DC antigen presentation machinery and T cell co-stimulatory molecules as well as cytokine secretion are

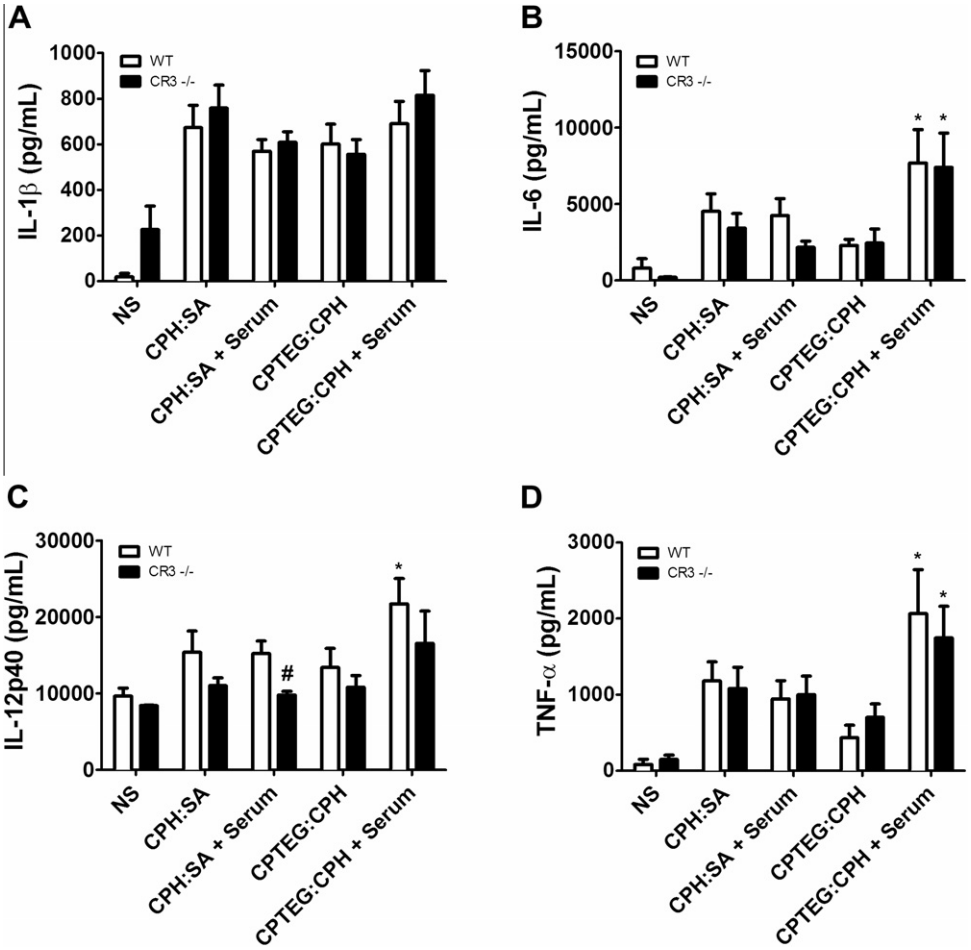

Fig. 7. Similar cytokine secretion profiles between WT and CR3$^{-/-}$ DCs upon co-culture with polyanhydride particles. After incubation with either sham- or serum-coated microparticles for 48 h WT (□) and CR3$^{-/-}$ (■) DC culture supernatants were harvested and assayed for (A) IL-1β, (B) IL-6, (C) IL-12p40, and (D) TNF-α. Data are expressed as the means ± SEM of three independent experiments performed in triplicate. LPS was used as a positive control stimulant with mean cytokine secretion values of 1041 pg ml^{-1} for IL-1β, 44,329 pg ml^{-1} for IL-6, and >50,000 pg ml^{-1} for both IL-12p40 and TNF-α. *Statistically significant difference from NS cells at $P < 0.05$. #Statistically significant difference between sham- and serum-coated particles within a chemistry at $P < 0.05$.

important and desirable characteristics of vaccine adjuvants that promote efficient naïve T cell activation and B cell differentiation [1,2]. A polymer chemistry-dependent enhancement of the expression of MHC II and co-stimulatory molecules (i.e. CD40 and CD86) and secretion of cytokines was observed in this study (Figs. 4–6), and is consistent with previous observations [9,14,34,35]. While sham-coated 50:50 CPTEG:CPH particles enhanced the expression of MHC II, CD86, and CD40, sham-coated 50:50 CPH:SA particles increased the secretion of IL-6, IL12-p40, and TNF-α. Of interest, adsorption of serum proteins onto hydrophobic 50:50 CPH:SA particles resulted in greater expression of MHC II and CD40 (Figs. 4 and 5). While the enhanced expression of CD40 was identified to be directly dependent on particle internalization, as shown in Supplementary Fig. S1, the expression of MHC II was found to not be dependent on particle internalization. These observations support previous findings that demonstrated that internalization alone is not sufficient to enhance DC activation [3]. In contrast, serum protein adsorption onto amphiphilic 50:50 CPTEG:CPH particles increased the secretion of pro-inflammatory cytokines (i.e. IL-6, TNF-α, and IL-12p40) in comparison with sham-coated particles (Fig. 6). Therefore, chemistry-dependent adsorption of serum proteins influences the expression of key surface markers and the production of cytokines that are involved in DC maturation and antigen presentation.

Together these results indicate that polyanhydride particles are capable of inducing DC activation and, more importantly, that the polymer chemistry can be rationally chosen to induce a mature DC phenotype. Induction of this activated DC phenotype may benefit antigen processing and presentation because DC migration and interaction with T cells is associated with activated DCs. For example, the intrinsic characteristics of the amphiphilic 50:50 CPTEG:CPH particles induced DC maturation in the absence of serum (i.e. enhanced MHC II, CD86, and CD40 expression), while serum proteins uniquely adsorbed onto the hydrophobic 50:50 CPH:SA particles and enhanced their adjuvant properties. These observations on the effect of protein adsorption phenomena on particle fate and interaction with immune cells will increase our understanding of the in vivo performance of these particles as vaccine adjuvants and/or antigen carriers. The specific interactions of particles with serum proteins and the consequences of their adsorption on the molecular interactions with DCs present an intriguing opportunity to rationally design efficacious vaccine delivery platforms.

5. Conclusions

In this study the profile of serum proteins adsorbed onto the surface of polyanhydride particles was influenced by polymer chemistry and elicited differential effects on DC activation. We also observed that complement receptor C3-mediated pathways were involved in the internalization of polyanhydride microparticles

by DCs regardless of the presence of serum proteins, highlighting the intrinsic pathogen-mimicking characteristics of these particles. The receptor-mediated internalization induced by either the direct interaction of adsorbed opsonins or the inherent pathogen-mimicking patterns of polyanhydride particles with CR3 may be exploited to design efficacious vaccine delivery vehicles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.06.001.

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